MEMORANDUM

SUBJECT: Ecological Hazard Report for TERA R-18-0001

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DATE: October 22, 2018

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I. SUMMARY

The recipient alga is *Chlorella sorokiniana* DOE1412 (Peñalva-Arana, 2018). The produced strain is PACE_Cs1412_SNRK2.

The aim of the genetic modification and subsequent field experiment is to: **1)** evaluate the translatability of GM phenotypes from a lab to an outdoor setting, and **2)** to compare the resistance compare the subject and recipient microorganisms when cultivated in outdoor miniponds with respect to their ability to produce biomass, and **3)** also to identify any increase or decrease in biomass productivity under biotic (bacteria, predators) and abiotic (diurnal temp and light) stressors from the environment. Based on the ubiquitous nature of *Chlorella* and the genetic modifications made to this strain, it poses **low** ecological hazard.

II. INTRODUCTION

EPA received a TERA from Arizona State University on behalf of the Producing Algae for Co-products and Energy (PACE) Consortium to produce one intergeneric eukaryotic algal construct, PACE_Cs1412_SNRK2, for field trial. The introduced intergeneric DNA gene present in the final construct encodes for SNF (sucrose non-fermenting) related kinase 2 (SNRK2) and was isolated from *Picochlorum soloecismus*. This gene was not codon optimized and was synthesized in its native state (coding regions only).

The gene is regulated by the endogenous *psaD* (a photosynthesis-related gene) promoter and terminator of *C. sorokiniana*. In addition, an actin promoter/terminator pair, which is endogenous to the

recipient microorganism, is used to regulate and drive the expression of the *Streptoalloteichus hindustanus* Sh *ble* gene, which confers zeomycin resistance to the recipient.

The final construct (SNRK_PACE_Chlorella_Plasmid) was introduced by electroporation into the recipient *C. sorokiniana* DOE1412. The DNA was randomly integrated by non-homologous insertion/recombination. The intergeneric gene product SNRK2 is expressed in the cytosol and is expected to confer *C. sorokiniana* DOE1412 with improved starch accumulation, growth, and photosynthetic efficiency.

III. TAXONOMY AND CHARACTERIZATION OF MICROORGANISM

A. Recipient Microorganism

The taxonomy and characterization of the donor, recipient, and subject microorganism was analyzed in the Taxonomic Identification Report for R-18-01 (Penalva-Arana, 2018).

USEPA has received a designated non-CBI TERA application similar to one received last year, case [redacted], for the same species *Chlorella sorokiniana* but for a different strain, this time *C. sorokiniana* PACE_Cs1412_SNRK2 (from now on known as PACE_Cs1412_SNRK2).

The submitter identifies the parental organism as *Chlorella sorokiniana* DOE1412. This strain was isolated from the field by Juergen Polle in 2013 (UTEX website, accessed 09/2018) and deposited to the CUNY collection. Subsequently, the National Alliance for Advanced Biofuels and Bio-Products (NAABB) consortium, after a screening process has made 30 of their best performing strains, including DOE1412, made this strain available to the public through UTEX. These UTEX strains have been well characterized by DOE for lipid production and growth kinetics. UTEX and DOE, describe the strain as a high temperature freshwater strain (cold-sensitive) with a maximum growth temperature of 42° C. The strain is also referenced as DOE1412, NAABB 1412 and NAABB 2412.

1. The Genus

The *Chlorella* genus has been previously assessed in a TERA submission ([redacted]). The genus was first delineated by Beyerinck in 1890. A comprehensive description of the genus *Chlorella* was first addressed by Shihira and Krauss (1965), in response to the lack of a sound taxonomic framework from which to base the identity of over 41 isolates known at the time. In 1976, Kessler identified 77 strains across 12 taxa, based on physiological and biochemical properties. Since then the genus has been found to have few useful diagnostically morphological characteristics, making it difficult to identify under a light microscope alone, and only through more rigorous methods can it be clearly classify as belonging to a specific species (i.e. DNA analysis) (Bock et al., 2011; Zou et al., 2016). Therefore, a more robust framework, based on polyphasic taxonomic approaches, has been developed to describe well over 100 potentially different *Chlorella* species (Bock et al., 2011; Zou et al., 2016). Based on integrative or polyphasic taxonomy a new system has been established which differs completely from the traditional artificial system of *Chlorella* and its relatives based on morphology alone. With the introduction of chemotaxonomy to *Chlorella* and other taxa our understanding of the taxonomy of *Chlorella* has changed radically. Based on SSU- and ITS rDNA sequences and light microscopy observations, Various publications have demonstrated how the high level of cryptic diversity found within *Chlorella*; and the polyphyletic characters between *Chlorella* and *Dictyosphaerium*, has resulted in numerous taxonomic revisions of these organisms (Zou et al., 2016). For example, Bock et al. (2011) detected six lineages of

Dictyosphaerium-like strains that are closely related to *Chlorella vulgaris* and described several new species. Krienitz et al. (2015) also attempted to demonstrate that the *Chlorella* species has been widely misclassified when using traditional morphological classification schemes, and suggested that only three 'true' spherical species belong to this genus: *Chlorella vulgaris*, *C. lobophora*, and *C. sorokiniana*. Based on biochemical and molecular data, the Chlorella genus was even more recently proposed to consist of five "true" Chlorella species (Zou et al., 2016). The number of Chlorella species appears to have reached ~14 with the inclusion of several former Dictyosphaerium strains (Bock et al., 2011), with suggestions of still others possible ones (Zou et al., 2016). Regardless of the ongoing debate of the number of species, *C. vulgaris* is considered the type species of this genus (Shihira and Krauss, 1965) and *C. sorokiniana* has retained authentic species status throughout these various taxonomic revisions, and is accepted as the recipient species.

Chlorella is a single-celled coccoid photosynthetic green microalgae, typically small (1-10 µm in diameter) and can be found either as singly or clustered in aquatic and terrestrial systems. It is found within the Chlorellaceae clade, in the class Trebouxiophyceae, in the order Chlorellales (Huss et al., 2009). In the past, some *Chlorella* species have been attributed to the Chlorophyceae, but true *Chlorella* belong to the Trebouxiophyceae. *Chlorella* sensu stricto is now placed explicitly in the class Trebouxiophyceae. This class also contains most of the known green algal endosymbionts, living in lichens, unicellular eukaryotes, plants and animals (Blanc et al., 2010). Members of the true *Chlorella* genus are also nonmotile with a single chloroplast and a rigid chitinous cell wall, characterized by glucosamine as a major component of the cell wall (Takeda 1991). These cells do not have mucilagineous envelopes or other cell wall ornamentation. They contain a single chloroplast with a pyrenoid. The pyrenoid is covered by a starch envelope and traversed by thylakoid membranes. Planktonic Chlorellaceae evolved into distinct forms, while terrestrial members exhibit morphological convergence, characteristic of the true *Chlorella* clade (Bock et al., 2011). Luo et al. (2010) state that in the traditional context, and also according to the first studies that included molecular and phylogenetic investigations, members of the genus *Chlorella* represent the archetype of a green spherical cell propagating purely by autosporulation (Huss et al., 1999). *Chlorella* has only been observed to reproduce asexually by nonmotile reproductive cells (autospores) that rupture through the mother cell. However, Blanc et al. (2010) reported that although *Chlorella* has long been assumed to be asexual, the genome of *C. variabilis* NC64A possesses genes encoding meiosis-specific proteins, and they also found homologs of the *Chlamydomonas* gametolysin proteins that promote disassembly of the gametic walls and allows for gamete cell wall fusion. Blanc et al. (2010) therefore suspect that meiosis and sexual reproduction are part of the *Chlorella* life cycle, that may have been simply overlooked, like the cryptic sex later identified in other algae species.

The submitters provided the following information to support the assignment of DOE1412 to *C. sorokiniana* (R-18-01):

"The recipient strain for this project will be *C. sorokiniana* DOE1412. This organism can be identified by running a whole cell approach to PCR with the specific primers developed for allowing discrimination from other *Chlorella* sp., even specific strains within species. The *C. sorokiniana* 1412 specific primers are a) FWD 5' GCGAAGAAGAAAATGTAAACTTATTAG 3' and b) Rev 5' CCATTCCAGTAATTGCTAAATCA 3'."

As demonstrated in the literature, *C. sorokiniana* can be distinguished from other Trebouxiophyceae using the internal transcript spacer 2(*ITS2*) gene sequence (Neofotis et al., 2016), and by comparison of the chloroplast genomic DNA (Lemeiux et al., 2014). Of note and with respect to Figure 1 of the TERA application, Rosenberg et al. (2014) used strains of *C. variabilis* that cluster within

the group that some suggest are the true *Chlorella*. The tree provided shows that *C. sorokiniana* clusters separately from *C. vulgaris* and *C. variabilis* strains, its closest neighbors in that study.

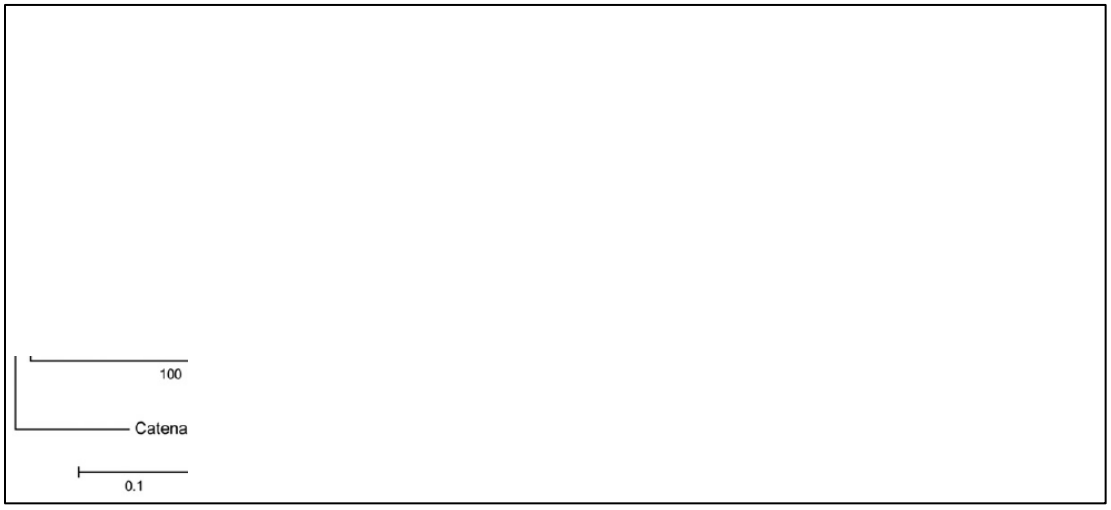

Figure 1. *C. sorokiniana* phylogeny (taken from TERA submission R-18-0001).

2. The Species

The species *Chlorella sorokiniana* has been previously assessed in a TERA submission ([redacted]. *Chlorella sorokiniana* is a unicellular, green alga that has been used as a model organism for photosynthesis studies and in various practical applications in agriculture, biotechnology, and as a food additive. Members of the Genus *Chlorella* have small, spherical or ellipsoidal cells (Figure 2) and are globally distributed; naturally occurring on soil and in freshwater. Strains of *C. sorokiniana* are generally observed to be non-flagellate cells but contain a vestigial flagellar apparatus. Sexual reproduction has not been reported in the literature to date but the submitter proposes it does occur and can be induced in the laboratory ([redacted]), thus, reproduction is often described as achieved by producing non-motile asexual autospores. Most *Chlorella* have a polysaccharide cell wall containing a sporopollenin-like substance that occurs in the walls of the pollen grains of higher plants ([redacted]).

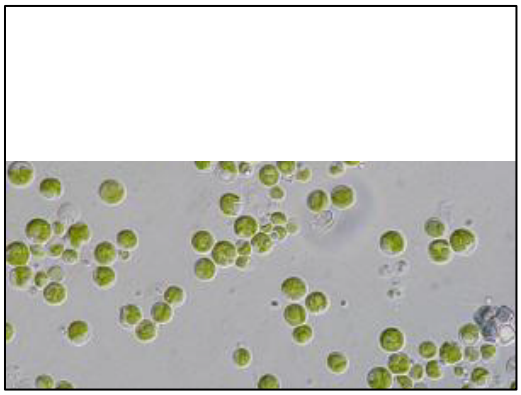

Figure 2. *C. sorokiniana* is a single celled non-flagellates microalgae (taken from TERA submission R-18-0001).

B. Donor Microorganisms

The intergeneric gene used to develop the strains in this TERA, the *SNRK2* gene was derived from a *Picochlorum soloecismus* strain, a genus of green algae in the class Trebouxiophyceae. *Picochlorum soloecismus* is a halotolerant, fast-growing and moderate lipid producing microalga that has been evaluated as a renewable feedstock for biofuel production by the DOE (Gonzalez-Esquer et al., 2018).

1. Sucrose Non-Fermenting (SNF) related kinase (*SNRK2*) gene

The sucrose non-fermenting (snf) related kinase 2 gene, *SNRK2*, is part of the serine/threonine kinases (Kertesz et al., 2002) and plays a key role in sugar metabolism in plant and animal kingdoms and controls multiple growth and metabolic processes.

Members of the sucrose non-fermenting related kinase Group2 (SNRK2) subclasses have been studied primarily in plants (e.g., *Arabidopsis*) and are implicated in both direct and indirect abscisic acid (ABA) response pathways dealing with environmental stress-signaling (Holappa et al., 2017; Todaka et al., 2015). Although the ABA signaling pathways have been extensively studied in plants, knowledge of their roles in algae and other lower photosynthetic species (e.g., cyanobacteria and lichen) remain limited. ABA synthesis in algae however is known to be induced by environmental stressors like drought or salt stress (Hartung, 2010). It has been shown that all SNRK2 subclasses are well conserved among higher plants, yet SNRK2s in algae (e.g., *Chlamydomonas*) have been classified as having distinct sequences from those found in higher plants (Hauser et al., 2011).

When overexpressed in Arabidopsis, *SNRK2* conferred increased sucrose synthesis, starch synthesis, and leaf growth (Zheng et al., 2010). The SNRKs have also been detected in almost all streptophyte algae (de Vries et al., 2018), and implicated with cold stress adaptation for the alga *C. reinhardtii* (Valledor et al., 2013). Streptophyte algae are a small group of freshwater algae ranging from scaly, unicellular flagellates (*Mesostigma*) to complex, filamentous thalli with branching, cell differentiation and apical growth (Charales). Streptophyte algae and embryophytes form the division Streptophyta, whereas the remaining green algae are classified as Chlorophyta (Becker and Marin, 2009).

The gene was synthesized in its native state (only the coding regions) without codon optimization and cloned into the PACE_Chlorella_Zeocin_Plasmid vector. This plasmid was specifically developed for use in genetically modifying green algae/*Chlorella*, and have been used for many years by PACE, including in a previous TERA submission [redacted]. The regulatory elements used to express the *SNRK2* gene are the *psaD* (a photosynthesis-related gene) and actin promoters and terminators, both of which are endogenous to the recipient microorganism. The submitter expected the overexpression of *SNRK2* would improve starch accumulation and growth in *Chlorella* cells. Compared to wild-type *C. sorokinia* 1412, the subject microorganism Cs1412_SNRK2 showed improved photosynthetic efficiency and growth (biomass).

C. Submission Microorganism

The *SNRK2* gene is expected and was shown to help the proposed strain, PACE_Cs1412_SNRK2 have better growth and photosynthetic efficiency than wild-type *C. sorokiniana DOE1412*. The submitters

report a 26-30% increase in growth (low to high light), along with a 21% increase in total carbohydrate accumulation in PACE_Cs1412_SNRK2 compared to wild-type *C. sorokiniana DOE1412 (Figure 3).*

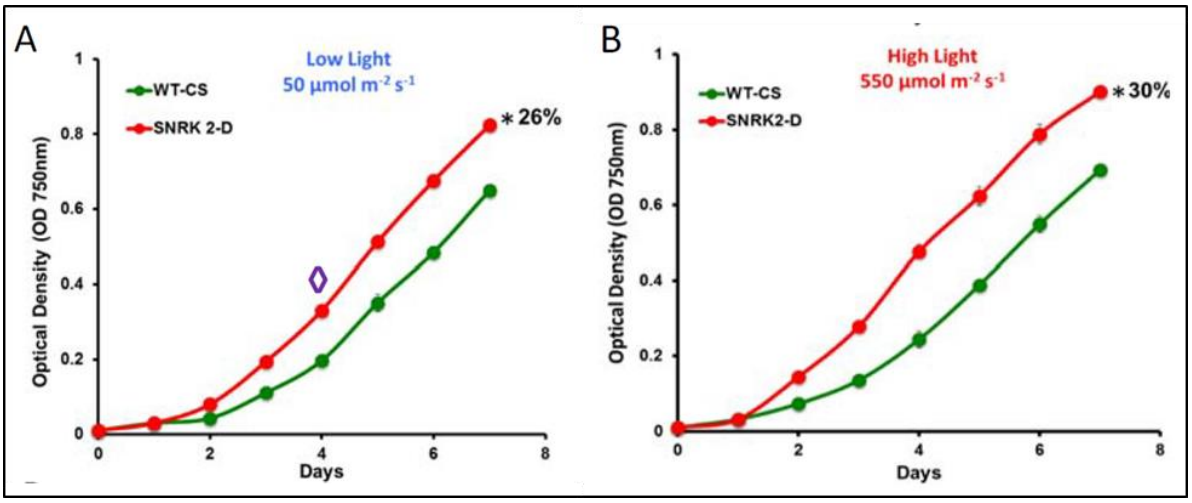

Figure 3. Photoheterotrophic growth of SNRK2 vs WT *C. sorokiniana* (R-18-01 Submission).
(A) Low light intensity. (B) High light intensity. Growth was measured at 750nm. These experiments were performed in triplicates. * indicates significant increase in growth.

The growth characteristics of *Chlorella sorokiniana* has been extensively studied due to its high performance in various factors (biomass, lipids, growth rate, and temperature tolerance) (Sayre et al., 2015). Many indoor/outdoor growth studies have been performed with this strain in attempts to optimize its productivity in various biotechnology fields. The genetic modifications presented for this submission enhances the growth and biomass accumulation, , which can be viewed as increase in its competitive advantage in the environment as it will consume more nutrients at a faster rate than that of the wild type recipient.

However, the survival characteristics are not expected to drastically change from the wild type recipient to the submission strain. Although the introduced genetic material does enable faster growth, it does not enable PACE_Cs1412_SNRK2 to survive in environments not tolerated by the wild type strain. In addition, the introduced *SNRK2* gene does not enable the submission strain with the ability to utilize any new or different substrates, nor does it impart any invasive properties.

Furthermore, the traits in PACE_Cs1412_SNRK2 are not new to the genus since increased growth and biomass accumulation have also been attained in wild type *C. sorokiniana* by tuning various growth parameters, which was reviewed by De Francisci et al. (2018). Table 1 shows that by adjusting basic growth parameters, researchers can tune wild type *C. sorokiniana*'s growth rate, lipid content, FAME yield, and protein content.

Table 1. **Characterization of *Chlorella sorokiniana* growth and biomass reported in literature. (modified from De Francisci et al., 2018)**

Research focus	Growth performance (d^{-1}/g L^{-1} d^{-1})	Lipid content (%, w/w)	FAME yield (%, w/w)	Protein content (%, w/w)	Reference
Effect of temperature	-	~10%	1.3–6.1%	-	*Patterson, 1970*
Effect of C/N ratio	-	13–46%	2.1–7.3%	-	*Chen and Johns, 1991*
Pigment composition	5.76 d^{-1}	10.00%	-	68.50%	*Matsukawa et al., 2000*
Effect of biochemical stimulants	42 mg L^{-1} d^{-1}	5–7%	-	45–60%	*Hunt et al., 2010*
Mixotrophic growth	0.44 d^{-1}	20–50%	-	10–32%	*Wan et al., 2011*
Effect of inoculum size	0.89 d^{-1}	-	-		*Lu et al., 2012*
Photoautotrophic/ heterotrophic growth	-	21–26% (P)	0.6–0.8% (P)	12–13% (P)	*Wan et al., 2012*
		20–56% (H)	12–33.6% (H)	6.2–13% (H)	
Cultivation with deep sea water	176.6 mg L^{-1} d^{-1}	51.70%	47.51%	-	*Chen et al., 2013*
Cultivation in cattle manure	12.77 mg L^{-1} d^{-1}	25–35%	12%	34%	*Kobayashi et al., 2013*
Fed-batch cultivation	3.29 d^{-1}	14.5–38.7%	12.8–34.1%	-	*Zheng et al., 2013*
Photoautotrophic/ heterotrophic/ mixotrophic growth	0.68 d^{-1} (P)	-	9.0% (P)	-	*Li et al., 2014*
	2.07 d^{-1} (H)		6.2–17.6% (H)		
	3.40 d^{-1} (M)		13.4–34.7% (M)		
Cultivation in domestic wastewater	220 mg L^{-1} d^{-1}	48.31%	-	-	*Ramanna et al., 2014*
Mixotrophic growth	1.602 d^{-1}	20–27%	-	-	*Juntila et al., 2015*
Effect of nitrogen limitation	3.21 d^{-1}	20–51%	-	-	*Li et al., 2015*
Continuous cultivation	2.41 d^{-1}, 1.52 g L^{-1} d^{-1}	-	6.24%	38.80%	*De Francisci et al., 2018*

Note: P, photoautotrophic; H, heterotrophic; M, mixotrophic.

IV. HISTORY OF USE

The history of use of Chlorella has been previously accessed in a TERA submission ([redacted]). *Chlorella* has a long history of research and experimentation, as it is a ubiquitous genus that can be found in marine, freshwater and edaphic habitats; making it one of the most ubiquitous and famous

microalgae genus worldwide. Much of what was first discovered about the fundamentals of photosynthesis and inorganic nutrition came from experiments using *Chlorella* (Shihira and Krauss, 1965).

There is no history of use of *Chlorella sorokiniana* for biofuel production. However various *Chlorella* species, including *C. sorokiniana*, have been extensively researched for their application in feed, food, nutritional, cosmetic, pharmaceutical and biofuels (Kang et al., 2004). *C. sorokiniana* has been researched as a health food due to its high carotenoid content and various vitamins (Cordero et al., 2011). *Chlorella* is not only a good genus for basic research but also a powerful superfood and has been proposed as a significant player in the development of second generation biofuels and medical treatments (Kumar et al., 2015; Pienkos and Darzins, 2009).

V. GENETIC MODIFICATIONS

1. *SNRK2* Gene

The subject microorganism has the sucrose non-fermenting (SNF) related kinase (*SNRK2*) gene from *Picochlorum soloecismus* randomly integrated into its genome, and is expressed using the *psaD* (a photosynthesis-related gene) and actin promoters and terminators, both of which are endogenous to the recipient microorganism. A brief history of the *SNRK2* gene in literature was discussed in section III.B.1

The *SNRK2* gene (non-codon optimized) was synthesized in its native state (coding regions only) and cloned into the PACE_Chlorella_Zeocin_Plasmid vector by Genewiz (https://www.genewiz.com/en). The PACE vector was developed by researchers at the New Mexico Consortium to introduce genes of interest into *Chlorella* sp. and is built on the *E. coli* vector backbone from plasmid pSL18.

2. Zeocin - Antibiotic Resistance Gene

The zeocin antibiotic resistance gene has been previously accessed in a TERA submission ([redacted]. Zeocin is a popular broad-spectrum antibiotic, effective for the selection of vectors bearing the *Sh ble* gene in a variety of cells, including bacteria, eukaryotes, plants and animals. The *Sh ble* gene was isolated from *Streptoalloteichus hindustanus* and is a small gene, only 370 bp in size, whose product inactivates zeocin. Zeocin is a copper-chelated glycopeptide antibiotic belonging to the bleomycin family of antibiotics and one of the phleomycins produced by several *Streptomyces sp*. Zeocin causes cell death by intercalating into DNA and cleaving it. The action of zeocin is effective on most aerobic cells. Typically, mammalian cells are sensitive to zeocin concentrations of 50-400 µg/ml, and bacteria to 25 µg/ml (Zeocin TDS, version #16D20-MM).

Zeocin belongs to the bleomycin (BLM) family of antibiotics, which have been widely used as chemotherapeutic agents for the treatment of skin, head and neck carcinomas. BLMs damage DNA directly, and some iron complexed BLMs have been reported to cause sequence-specific DNA cleavage in the presence of oxygen. BLMs are harmful to any cells that come in contact with them, including the BLM-producing organisms, hence why producing cells also produce proteins that can modify and sequester them, including the *Sh ble* protein found in *Streptoalloteichus hindustanus* (Miyazaki et al., 2009). Although BLMs have not been used as antibacterial agents (not approved for human use), many clinically isolated strains of methicillin- resistant *Staphylococcus aureus* (MRSA) produce BLM-binding

proteins that sequester these antibiotics, leading these strains to be resistant to BLMs at high levels. Other BLM-binding proteins has been found in *E. coli* (Miyazaki et al., 2009). Due to its wide distribution across clinical and environmental strains, the use of zeocin resistance markers used in this TERA's strains should not be considered an antibiotic resistance threat.

VI. POTENTIAL ECOLOGICAL EFFECTS OF THE RECIPIENT MICROORGANISMS

The interactions of algae in aquatic and terrestrial environments and their role in aquatic food webs were discussed in a previous risk assessment for an algal submission by McClung (2013).

A. Aquatic Ecosystems

A number of factors affect the rise and fall of algal populations in the aquatic environment including the physical factors of light, temperature, weather, water movements, flotation, the chemical nutrient status of nitrogen, phosphorus, silicon, calcium, magnesium, potassium, sulfate, chloride, iron, manganese, and other trace elements, and organic matter (Ikawa, 2004). There are a number of biological factors as well including the presence of resting stages, predation, and parasitism. The polyunsaturated fatty acids produced by algae can affect algal growth. In addition, a number of biological substances are known to be produced by algae that inhibit the growth of other algal or of zooplankton grazers, as shown by Pratt (1944; Pratt et al., 1945). Likewise, it has been shown that some algae detect "infochemical" signals from grazers, and can change their morphology accordingly to try to avert predation (Lass and Spaak, 2003). Food webs in water bodies are complex and dynamic and have been shown to vary from season to season and with other perturbations of the water body. e.g., eutrophication (Lindeman, 1942; Martinez, 1991).

B. Aquatic Food Webs

Algae and cyanobacteria are the basis of the food web in both freshwater and marine aquatic ecosystems. The phytoplankton community of a typical north-temperate lake has been shown to consist of up to several hundred algal species that co-exist (Kalff and Knoechel, 1978). Phytoplankton diversity is influenced not only by the different ecological niches within a water body (e.g., benthic vs. pelagic regions), but also by a number of temporal and spatial variations in factors such as nutrient supply, temperature, dissolved oxygen, predation, and parasitism (Wehr and Sheath, 2003; Townsend et al., 1998). Nutrient supply and herbivory are thought to be the most important parameters affecting diversity changes over time. According to Wehr and Sheath (2003), the phytoplankton species composition in lake food web ecosystems is important because the 'functional properties of algal assemblages vary strongly with species composition'. Different taxa are important because features that are sometimes used to classify various species such as photosynthetic pigments, storage products, motility, reproduction, cell ultrastructure, and even DNA sequence have functional importance. For example, nitrogen fixation ability is of great functional importance but is restricted to a limited number of cyanobacteria. Also, photosynthetic pigment production is important, for instance with the red accessory pigment phycoerythrin which has an absorption maximum of 540-560 nm. The presence of this pigment broadens the photosynthetic capacity of an ecosystem by facilitating growth at greater depths (Goodwin, 1974). Autotrophic picoplankton have a strong competitive advantage under phosphorus-limiting conditions (Suttle et al., 1988; Wehr, 1989).

Diversity in the size fractions of phytoplankton is an important aspect of algal communities and thus food webs. For planktonic food webs, cyanobacteria have a dominant role in aquatic productivity. It is

these smaller autotrophs that provide excreted dissolved organic compounds that provide substrates for heterotrophic bacterial growth. In addition, cyanobacteria are directly grazed by protozoa (microflagellates and ciliates). This microbially-based food web in which the major portion of autotrophic production occurs is important to the marine food webs. The microbial food web consists of those organisms that are < 1000 µm, and in freshwater benthic ecosystems consists of (presented by increasing size fraction) cyanobacteria and bacteria, followed by microflagellates, diatoms and green algae, which are then consumed by ciliates, rotifers, copepods, oligochaetes, nematodes, and then invertebrate macrofauna followed by the larger vertebrates (Bott, 1996). A complex microbial food web has bacteria and algae at the lowest trophic level, which are then consumed by protozoa and meiofauna. Meiofauna are organisms in the size range of approximately 50 - 1000 µm and includes large ciliates and metazoan (e.g., rotifers, copepods, and oligochaetes).

An important link between microbial food webs and classical food webs are with the autotrophic picoplankton (> 0.2 - 2 µm). These cyanobacteria are grazed mainly by micro-zooplankton (ciliates, flagellates) rather than by cladocerans or copepods (Pernthaler et al., 1996; Hadas et al., 1998). Size affects the sinking rate with smaller planktonic species sinking more slowly. Thus, the smaller species remain more prevalent in the euphotic zone.

C. Terrestrial Ecosystems

Algae occur in nearly all terrestrial environments on earth and are invariably encountered on and beneath soil surfaces (Metting, 1981). Acceptance of algae as bona fide soil microorganisms evolved late in the 19th century when it was recognized that certain groups were restricted to soil, including some *Chlorella* species (Shihira and Krauss, 1963; Kessler, 1976). Over 38 prokaryotic genera and 147 eukaryotic genera have been identified as terrestrial species, the majority of which are truly edaphic (i.e., soil). As expected solar radiation, water and temperature are the most abiotic factors controlling their distribution, metabolism and life histories (Metting, 1981). Biotic interactions are also important, but much well less understood. Algae play an important role in primary and secondary plant community succession by acting as an integral part of ecosystem. Algal communities living in soil have the principal function of being primary producers, nitrogen fizxation, and stabilization of aggregates (i.e. can even prevent soil erosion) (Metting, 1981). Algae concentrations is soils are typically found to be between 10^3 and 10^4 cells/gram but have been reported as high as 10^8 (Metting, 1981).

D. Dispersal of Algae in the environment

As reviewed by Tesson et al. (2016), microalgae have been reported across a wide range of ecosystems, covering almost all latitudes from tropical to polar regions. Due to their relatively small size (few to 500um), microalgae are dispersed by water, air, and various biotic vectors (e.g., humans and animals) (Kristiansen, 1996b; Tesson et al., 2016). These mechanisms and organisms of dispersal were discussed in a previous algal risk assessment by McClung (2013).

I. Dispersal by Water

Passive dispersal of algae by water can occur wherever there is running water between connected water bodies. A study by Atkinson (1988; as cited by Kristiansen, 1996b) found that the colonization of a newly constructed reservoir was from the inflow. It was several years later before the appearance of organisms other than those found in the catchment area. Heavy precipitation and flooding can result in

algal dispersal by connecting water bodies that are usually isolated. Algal dispersal by water is likely more important in wetter environments that in arid regions.

II. Dispersal by Aerosols

Air is an important dispersal mechanism of algae, and it is thought that algae have spread throughout the globe as aerosols. As early as 1844 Ehrenberg recognized the presence of airborne algae in dust samples collected 300 km off the nearest coast by Darwin in 1939 on the H.M.S. Beagle (as cited by Kristiansen, 1996b).

According to a review article by Sharma et al. (2007), "In general, bioaerosols range from 0.02 to 100 µm in diameter and follow the same physical rule as any particle of a similar aerodynamic diameter. They disperse via air movements and settle according to the settling velocity, available impaction, surface, and climatic factors prevailing in the area (Burge and Rogers, 2000). Air movements within a laminar boundary layer surrounding the source usually release such particles. Many of the particles remain in the layer and eventually settle near the source (<100 m), while some are carried aloft with turbulence and transported by the wind over a long distance. The processes responsible for the release and atomization of bioaerosols from natural sources are as follows:

1. Sweeping of the surface or rubbing together of adjacent surfaces by wind and gusts dislodges the bioparticles from the surface. Dried algae caught by the wind are carried away like dust particles (Grönblad, 1933; Folger, 1970).

2. Formation of oceanographic aerosols by wave action and the bursting of bubbles at the water-air interface (Woodcock, 1948; Stevenson and Collier,1962; Maynard, 1968b; Schlichting, 1974). Fragments of scums and foams with algal contents along the shoreline of water bodies can be picked up by the wind and carried aloft (Maynard, 1968b).

3. During heavy rainfall, algae are splashed up by raindrops and can be entrained into the atmospheric air by thermal winds (Burge and Rogers, 2000).

4. Storm activity over land and sea where great turbulence is experienced.

5. Human activities, such as agricultural practices, construction and maintenance practices, sewage treatment plants (Mahoney, 1968, as cited in Sharma et al., 2007), garbage dumping, highway traffic, and to a limited extent weapons testing and spacecraft launching, can result in the atomization of constituting algae (Schlichting, 1974; Kring, 2000).

6. Atomization of aerosols to a low height also occurs when surface water containing blooms is used for irrigation and recreational activities like boating, jet skiing, and so forth. (Benson et al., 2005)".

Sharma et al. (2007) also stated, based on the result of earlier publications, that green algae, cyanobacteria, diatoms, and tribophytes comprised most of the aero-algae flora. Cyanobacteria dominate the aero-algae flora of tropical regions whereas chlorophytes (green algae) dominate in the temperate regions.

Brown (1964) conducted studies on airborne algae using agar petri dishes suspended in stationary locations in Texas, and impaction studies of algae onto agar petri dishes from moving automobiles in 14 states. He also collected samples from an airplane. The impaction from the moving automobiles and planes yielded the greater numbers and diversity of algae. For example, the agar plates held from a moving car in Pennsylvania yielded 140 algal impactions composed of approximately 25 different genera of algae. A 10-second exposure obtained from a moving car sampling a local dust cloud resulting from plowing of a field recorded 5,000 algal compactions, of which 4,500 were chlorophycean or xanthophycean. *Chlorella* was one of the algal genera most frequently found, both in stationary dishes and impaction either by car or plane. The author stated that a large number of different genera and species can be transported in the air. The algal content of dust was quite high at > 3000 cells per m^3. The author concluded that soil is the predominant source of airborne algae.

Schlichting (1969) conducted studies on airborne algae in Michigan and Texas using Millipore filters and bubblers containing soil-water extracts at heights of 6, 15, 30, 75, and 150 feet from the ground. Also, aerial sampling of maritime algae was made from a ship 100 miles off the coast of North Carolina. Over an eight year period, the number of algae collected never exceeded 8 /ft^2. He then estimated that a person at rest would inhale 240 algal cells per hr, which would result in an inhalation exposure of approximately 2880 cells/day. Higher algae numbers were found in the Texas samples from dust than those from water environments.

The diversity and abundance of airborne green algae and cyanobacteria on monuments and stone art works in the Mediterranean Basin was studied by Macedo et al. (2009). Airborne *Chlorella* species were found in the top three frequently encountered chlorophyta isolated which were *Chlorella, Stichococcus,* and *Chlorococcum.*

The diversity of aeroalgae in a Mediterranean river-reservoir system was found to be high (Chrisostomou et al., 2009). They found that nanoplanktonic algae comprised the majority (46.4%) of the aero-algae flora. The predominant alga was the green alga *Chlorella.* Three of the most frequently isolated nanoplanktonic airborne algae were *Chlorella vulgaris, Didymocystis bicellularis,* and *S. obliquus.* The authors suggested that these vegetative cells have a protective external coating that allows them to resist desiccation in bioaerosols for short distances.

Genitsaris et al. (2011) did a comprehensive review of studies in the published literature on airborne algae. They summarized that the most frequently occurring algae isolated from aerosols were *Chlorella, Scenedesmus, Chlorococcum,* and *Klebsormidium,* and the cyanobacterium *Lyngbya.* These were found in more than 40% of the sites that had been sampled by various researchers in their aero-algae studies.

In aquatic habitats, microorganisms are known to be concentrated in the surface films and in foams on the water surfaces (Maynard, 1968). Schlichting (1974) conducted studies on the ejection of microorganisms into the air with bursting bubbles. He found that bubbling air through a bacterial culture resulted in 2,000 times more bacteria in the bubble jet droplets. Microorganisms in the range of 0.3 to 30 µm in diameter can be carried in atmospheric water droplets (Woodcock, 1948, as cited by Schlichting, 1974).

Airborne algae are subject to desiccation stress and ultraviolet light exposure (Sharma et al., 2007). Desiccation, the equilibration of an organism to the relative humidity of the surrounding atmosphere, is an intensive stress that typically, most phototrophic organisms cannot survive (Holzinger and Karsten,

2013). However, there are studies that suggest that some algae can survive desiccation stress (Evans, 1958, 1959; Schlichting, 1961). A comprehensive list of algae capable of surviving desiccation was published in 1972 by Davis. Parker et al. (1969) reported that various cyanobacteria and green algae survived desiccation as viable algae were found in decades-old air-dried soil samples. This is in contrast to Schlichting (1960) who reported survival of only four hours with desiccation stress. Ehresmann and Hatch (1975) studied the effect of relative humidity (RH) on the survival of the unicellular eukaryotic alga *Nannochloropsis atomus* and the prokaryotic alga *Synechococcus* sp. Viable cells of the latter species could be recovered at all the RHs tested (19,40,60,80, and 100%). However, there was a progressive decrease in the number of viable *Synechococcus* cells with lower RHs. There was a stable survival at RH 92% and above. The results with the eukaryotic green alga were very different. No viable cells of *N. atomus* were recovered below 92% relative humidity. In an earlier study Schlichting (1971) found that algae remained viable under a wide range of environmental conditions including RHs of 28-98%. The stress associated with atomization of the algae was responsible for rapid decrease in viability. So perhaps, the gradual air-drying of soil samples as in Parker et al. (1969) did not result in death of the microorganisms.

Recent work by Szyjka et al. (2017) has demonstrated that cultivation of genetically engineered (GE) algae in outdoor ponds can leads to the aerosol release of these organisms. Their data shows that algae grown in ponds can travel and be detected in bucket traps as a function of distance and wind direction. Using qPCR to detect both wildtype and the GE strain showed detectable levels in all traps at distances from 5-50 meters away. However, neither strain was able to outcompete local or airborne algae taxa in either the trap buckets or in experiments conducted using local eutrophic and oligotrophic lake water containing local taxa. Their research also showed that airborne algae have high diversity (species detected using ITS2 primers) and can invade any available waters, including members of the species being tested. This only reinforces the conclusion that aerophilous algae, such as *Chlorella*, can and will travel, both short and possibly long distances when grown in open ponds, and potential risks lie in an alga's ability to survive, establish and persist in the receiving environment. Additionally, the potential for horizontal gene transfer of the GE strains optimized genes is possible, as this same species or close relatives of this species, may be found in the surrounding environment, in both terrestrial and aquatic environments.

III. Dispersal by Aquatic and Terrestrial Organisms

Aquatic and terrestrial organisms are responsible for algal dispersal. Even fish can act as vectors. For example, numerous species of plankton algae including cyanobacteria, green algae, and diatoms have been found to pass undamaged through the digestive track of the plankton-eating gizzard shad (Velasques, 1940 as cited by Kristiansen, 1996b). Insects such as beetles have been found to carry viable algae in their digestive tract (Parsons et al., 1966, as cited by Kristiansen, 1996b), and thus, their faecal pellets can distribute algae to new water bodies. Milliger and Schlichting (1968) found 20 species of green algae in the intestinal tract of beetles. Algae dispersal by beetles is a likely mechanism for small water bodies for short distances (Kristiansen, 1996b). Other insects can disperse algae to various water bodies. Reville et al. (1967) found that with four species of aquatic Diptera (craneflies and midges), 21 different genera of algae were found on the collected insects. Likewise, Sides (1968) found that the mud dauber wasp was capable of carrying algae and protozoa as nine and four genera, respectively, were isolated from aseptically collected insects. Parsons et al. (1966, as cited by Kristiansen, 1996b) reported the presence of 20 genera of viable blue-green algae (currently cyanobacteria), green algae, and euglenoids in and on dragonflies and damselflies. Dragonflies are thought to be able to transport algae possibly long distances (Maguire, 1963).

Water-living mammals and other mammals such as mink, muskrats, and raccoons can transport viable algae on their fur and sometimes in their intestinal tracts. Human activities can also transport algae between water bodies. For instance, the use of felt-soled wading boots has been banned in a number of states as they have been shown to transport non-native larvae, spores, and algae between water bodies. In Vermont, the felt-soled wading boots are believed to have spread didymo, a slimy alga also called rock snot, to various rivers throughout the state. This alga forms dense mats that blanket the bottom of the stream like a shag carpet, changing pristine trout streams to a green, yucky mess, according to Shawn Good, a fisheries biologist with the state Fish and Wildlife Department (http://usatoday30.usatoday.com/news/nation/environment/2011-04-28-rock-snot-felt-sole-wader-ban_n.htm).

IV. Dispersal by Birds

Water birds are the most important vectors for algae dispersal as they can transport live algae on their feet and feathers and sometimes internally in their bills or in their digestive tract. Water birds such as seagulls have been shown to transport algae, particularly aquatic desmids, in wet mud on their feet for long distances (Strøm, 1926 as cited by Kristiansen, 1996b). Desiccation is of course of great importance with the viability of live algae transported on the feathers or feet of birds. Algae carried internally in the digestive tract are not subject to desiccation stress.

Migratory birds have a significant role in the transport of algae for long distances. Proctor (1959) studied the carriage of algae in the intestinal tract of numerous migratory bird species obtained from playa lakes in Texas and Oklahoma. A number of freshwater algae species were found in the alimentary canal of 25 different migratory birds. Algae were found in the lower digestive tract of the pied-bill grebe, the green-winged teal, the blue-winged teal, the shoveler, the American coot, the killdeer, the dowitcher, the American avocet, the Wilson's phalarope, and the belted kingfisher. Since many species of blue-green algae (currently cyanobacteria) and green algae do not have spores or specialized resting structures, the algae were assumed to have been transported as vegetative cells. Based upon the rate of movement of the algae through the alimentary tract and the flying speed of some common migratory birds, Proctor (1959) suggested that algae could be easily transferred between lakes 100 - 150 miles apart, with much greater distances possible with cells or colonies in the caecum of the birds.

Schlichting (1960) also investigated the transport of algae on and in various waterfowl. He measured the carriage of chlorophyta (green algae), cyanophyta (blue-green algae), chrysophyta (golden algae), euglenophyta, bacteria, fungi, protozoa, and rotifers and on the feet and feathers, and in the bill and gullet, as well as in the faecal matter of 105 birds representing the following 16 species of waterfowl: black duck (*Anas rubripes*), blue goose (*Chen caerulescens*), buffie-head duck (*Bucephala albeola*), Canada goose (*Branta canadensis*), coot (*Fulica americana*), Eastern belted kingfisher (*Megoceryle alcyon*), gadwall *(Anas strepera*), goldeneye (*Glaucinetta clangula americana*), green-winged teal (*Anas carolinensis*), mallard (*Anas platyrhynchos*), redhead duck (*Aythya americana*), ring billed gull (*Larus delawarensis*), ruddy duck (*Oxvura jamaicensis*), spotted sandpiper (*Actitis macularia*), common snipe (*Capella galinago*), and wood duck (*Aix sponsa*).

The field collection experiments demonstrated that the water birds retained viable forms of algae and protozoa both externally and internally. For those organisms carried externally on the feet and feathers, the birds exposed to the air for less than four hours carried a great variety of organisms. Those exposed to air for longer periods of time had fewer viable organisms. With eight hours exposure to air, there were some organisms on the feet of birds, but a greater variety was found to be carried in the

bills. The birds exposed to the air longer than eight hours yielded very few organisms. The contents from the gullets sampled produced good algal growth in culture, whereas only a few of the 163 faecal samples contained viable algae or other organisms. Viable organisms found on the waterfowl consisted of 86 species from the feet, 25 species from the feathers, 25 species from the bills, 14 species from the gullets, and 12 organisms from the faecal material.

The following species of green algae were found on the feet of the waterfowl: *Ankistrodesmus braunii, A. convolutus, A. falcatus, Arachnochloris-like cells, Arthrospira gomotiana, A. jenneri, Chlamydomonas globosa, C. mucicola, C. pseudopertyi, C. sp., Chlorococcum sp., Chlorella ellipsoidea, C. vulgaris, Chlorella sp., Closteriopsis-like cells, Dactylococcopsis acicularis, Franceia sp., Glenodinium sp., Gloeocystis gigas, Mougeotia sp., Nannochloris bacillaris, Oedogonium sp., Oocystis rorgei. Palmodictyon sp., Protococcus sp., Rhabdoderma irregulare, Rhizoclonium fontanum, Scenedesmus abundans, S. dimorphus. S. quadricauda, Scenedesmus sp., Sphaerocystis, Schroeteri, Tetraedron minimum, T. sisconsinense, Tetraedron sp.,* and *Ulothrix sp.*

The cyanobacteria found on the feet included the following species: *Anabaena affinis, Aphanocapsa sp., Aphanothece castagnei, A. nidulans, Chroococcus dispersus, C. minutus, Gloeocapsa sp., Gloeothece linearis, Lyngbya attenuata. L. limnetica, L. sp., Microcystis aeruginosa, Nostoc sp*.(?), *Oscillatoria angustissima, O. limnetica, O. subbrevis, O. tenuis, O. terebriformis, Oscillatoria sp., Pelo-gloea bacillifera, Phormidium mucicola, P. tenue, Phormidium sp., Plectonema nostocorum,* and *Synechococcus aeruginosus.*

Although much fewer numbers of green algae, cyanobacteria, golden algae, euglenoids, protozoa, and fungi were found on the feathers and bills, *Chlorella sp.* was found in both. It was also speculated by Schlichting (1960) that some microalgae, specifically *Chlorella*, may become embedded in the matrix of larger taxa, such as *Gloeocystis*, and be able to be transported away not only far but protected for greater periods of time.

E. Ecology of Recipient Microorganism

Three genera of green algae, *Chlorella, Chlamydomonas,* and *Scenedesmus* are the dominant green algae in many aquatic habitats and are frequently isolated from marine, fresh water, soils and air samples, as they can tolerate a wide range of environmental conditions (Trainor, 1998 as reference in J-13-0003). *Chlorella* is a simple airborne microalga, present in terrestrial and aquatic habitats, whose minute cell size and resistance against environmental stress allows for long-distance dispersal (Hodac et al., 2016). Chlorella is an aerophilous algae (found in air), a type of algae shown to have better adaptation and growth responses compared to their solely soil and aquatic counterparts (Sharma et al., 2007).

Chlorella is resistant against a number of environmental stressors, related to its metabolic versatility, and thus is able to cope with shortages of nutrients and water. This genus has a high tolerance to temperature and can easily live in both terrestrial and aquatic ecosystems. Members of the genus *Chlorella* are found in freshwater natural and artificial water habitats throughout the world (Trainor, 1998) and some species can even thrive in polar regions and hot deserts (Hodac et al., 2016). *Chlorella* have been reported from nearly all soil types, including: desert soil crusts, where it was one of the most common genera found across 4 of 7 different biomes sampled across the Namibian-Angola border (Budel et al., 2009); humic tropical soils in India, biofilms covering natural and artificial subaerial substrates and dwell in soils, and polar desert soils in Antarctica and Artic (Hodac et al. 2016). They can

be also grown in wastewater and used for the removal of metals (De-Bashan et al. 2008). Phylogenetic analysis (using SSU and ITS2 rDNA sequencing) has shown their polar, temperate and tropical distribution, in addition to demonstrating that even polar isolates are closely related to temperate ones (Hodac et al., 2016). Hodac et al. (2016) concluded based on sequence similarities that *Chlorella* might be capable of intercontinental dispersal; however, they acknowledge that their actual distributions may exhibit biogeographical patterns but requires further research. Although most *Chlorella* species are naturally free-living, some are known photosynthetic symbionts, such as one species known to be a symbiont of the unicellular protozoa *Paramecium bursaria* (Blanc et al., 2010).

Due to its high lipid content, compared to other algae (see Table 2), and its ability to growth under various growth conditions (see Table 2), *C. sorokiniana* has been studied extensively for wastewater remediation and production of biofuels. *C. sorokiniana* has been specifically sequenced by the National Alliance for the Advancement of Biofuels and Bioproducts (NAABB) consortium to increase collection of significant data for its rapid development of nuclear transformation systems (i.e., increase the ability of genetically engineer strains); also basic research on lipid production and biomass productivity has been conducted for various *Chlorella* strains. *Chlorella* has been identified as a candidate genus for development as a biofuel feed stock by the Department of Energy (DOE) (Sayre et al., 2015), as algal lipids are considered an ideal feedstock for transportation fuels (Pienkos and Darzins, 2009). Also, different *Chlorella* species prefer different growth temperatures, with *C. vulgaris* preferring 28-32˚ C while *C. sorokiniana* preferring temperatures from 36-42˚C and can even grow well at temperatures above 40˚C after a short adaptation period (De-Bashan et al., 2008).

When *Chlorella sp.* strain *DOE1412* was used as model strain by NAABB (Sayre et al., 2015) it was found that *Chlorella* exhibits much higher maximum specific growth rates at the optimal temperatures and greater thermal tolerance than other species. Therefore, *C. sorokiniana* strains that have been specifically engineered to withstand new stressors may be able to disperse and establish in previously uninhabitable niches, such as inland saline lakes and produced water ponds.

Table 2. Chemical composition of Algae – (% on dry matter basis) (from http://www.oilgae.com/algae/comp/comp.html – source Becker, 1994).

Strain	Protein	Carbohydrates	Lipids	Nucleic acid
Chlorella obliquus	50 - 56	10 -17	12 -14	3 - 6
Chlorella quadricauda	47	-	1.9	-
Chlorella dimorphus	8 -18	21 - 52	16 - 40	-
Chlamydomonas rheinhardtii	48	17	21	-
Chlorella vulgaris	51 - 58	12 - 17	14 - 22	4 - 5
Chlorella pyrenoidosa	57	26	2	-
Spirogyra sp.	6 - 20	33 - 64	11 - 21	-
Dunaliella bioculata	49	4	8	-
Dunaliella salina	57	32	6	-

Euglena gracilis	39 - 61	14 - 18	14 - 20	-
Prymnesium parvum	28 - 45	25 - 33	22 - 38	1 - 2
Tetraselmis maculata	52	15	3	-
Porphyridium cruentum	28 - 39	40 - 57	9 -14	-
Spirulina platensis	46 - 63	8 - 14	4 - 9	2 - 5
Spirulina maxima	60 - 71	13 - 16	6 - 7	3 - 4.5
Synechoccus sp.	63	15	11	5
Anabaena cylindrica	43 - 56	25 - 30	4 - 7	-

Microalgae, depending on specific species characteristics and culture conditions, will employ different metabolic pathways for growth. *C. sorokiniana* may be capable of growth under autotrophic, heterotrophic and mixotrophic conditions (Kim et al., 2013). Under autotrophic conditions microalgae fix CO_2 to organic matter using light energy, which results in the reduction of CO_2. Heterotrophic microalgae can grow using organic carbon a sole carbon source without the need for light. Mixotrophic microalgae can metabolize both organic and inorganic carbon using metabolic characteristics of both auto- and heterotrophs; using energy produced from organic sources for cell synthesis and storage of chemical energy converted from light energy (See Table 3). Requirements for nitrogen and phosphorus seem to also differ between all three growth types. For example, Kim et al. (2013) reported higher requirements under heterotrophic growth conditions than for auto- or mixotrophic growth conditions.

Table 3. Energy and carbon source of microalgae by growth type (adapted from Kim et al., 2013).

Growth type	Energy Source	Carbon Source
Autotroph	Light	Inorganic
Heterotroph	Organic	Organic
Mixotroph	Light and organic	Inorganic and organic

Autotrophic microalgae growth has been shown to be lower than that of heterotrophic or mixotrophic types; thus making it possible and advantageous to grow microalgae at high rates in lightless conditions that match or exceed autotrophic growth. Kim et al. (2013) demonstrated this to be true for *C. sorokiniana* (see Figure 4), with hetero- and mixotrophic conditions resulting in a 2-times higher growth rate. The most important factor for the growth of any autotrophic culture is light intensity, while organic carbon as a sole carbon source significantly affects the growth of heterotrophic microalgae. Therefore, Kim et al. (2013) suspected that the slower growth rate under autotrophic conditions was due to photo inhibition due to high cell density. This has been shown to be the case for *C. sorokiniana* when comparing heterotrophic versus autotrophic growth at high densities before (Zheng et al., 2012).

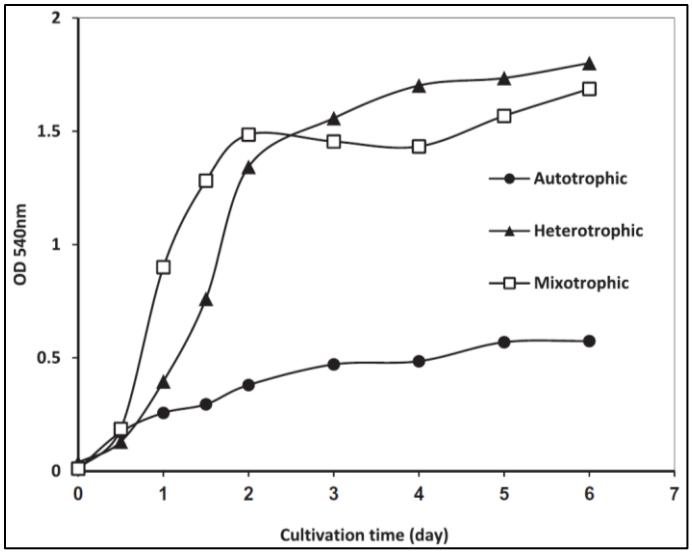

Figure 4. Growth of *C. sorokiniana* in auto-, hetero- and mixotrophic conditions (taken from Kim et al., 2013).

Its overall essential nutrient requirements do not differ greatly from that of other microalgae. Eyster (1966) reported on the most suitable growth conditions for *C. sorokiniana* when grown in a photobioreactor, and went on to develop an optimal medium for their growth at temperatures of 25 and 39˚C. He reported that minimum nutrient concentrations varied between 2-5 times higher at the higher temperature, except for phosphorus which required a concentration 25x higher, and it was hypothesized this might be related to the thermotolerant nature of *C. sorokiniana*. It was also noted that critical algae densities matched between the two temperatures. Eyser also reported that *C. sorokiniana* cultures require a minimum pH of 6.2, with maximum growth occurring in the 6.15-6.3 pH range. He also reported that when grown at higher densities *C. sorokiniana* is more tolerant of shifts in pH. His study gave no indication that *C. sorokiniana* requires B, Na, Co, Mo or chloride for growth.

The occurrence of many species of algae throughout the world suggests that algae can readily disperse over great distances. Studies on microalgae have shown that most species are globally distributed (cosmopolitan) but some species have more restricted distribution due to environmental factors such as temperature or humidity, and limited dispersal mechanisms (Kristiansen, 1996a). In a review of data on the distribution of coccid green algae in the environment, Komárek and Comas (1984) said that the distribution is dependent on the specific environmental requirements of the taxon. They stated that "Chlorococcalean algae (*Chlorella* belongs to this group) are traditionally supposed to be organisms of cosmopolitan occurrence. Many species occur, indeed, in various regions all over the world, but, many other taxa occur in geographically limited areas, mainly in either the northern or the tropical countries". This easily explains why *C. sorokiniana* has been collected from various biome types across Africa, for example: from the biological soil crusts across Africa's Kalahari's dry savanna, Namibia's succulent Karoo, and Zambesian dry forest (Budel et al., 2009), demonstrating its ability to survive in highly diverse ecosystems, with extreme temperature ranges. One strain of *C. sorokiniana* was reported to have an optimal growth at temperatures between 38-42˚C (Kessler, 1985) while others report that it can be grow at temperatures ranging from 14-38˚C (Patterson, 1970). Therefore, *C. sorokiniana* is very well equipped to survive in the desert where this TERA, if approved, will take place.

Chlorella has a few known predators that are of concern for open pond cultivation, among them rotifers and some bacteria. Various strategies are being investigated for loss prevention of *Chlorella*

cultures. Many are exploring the use of biomolecule production in algae for improving their innate defense against bacteria and rotifers (Sayre et al., 2015). Sayre et al. (2015) for example has examined the use of various antimicrobial peptides (AMPs) to protect against rotifer and bacterial infection and its effect on algae growth, including that of *C. sorokiniana*, while others are looking at genetic engineering endogenous compounds that can be produced and released by the various strains to prevent infection of the cultures. Cultivation pond experiments with *Chlorella* have demonstrated that algal-associated bacterial communities shift over time, and crashes of cultures are often associated with *Vampirovibrio chlorellavorus* infection. Therefore, various groups are working to develop PCR-based tools for monitoring contaminants. NAABB, for example, has designed primers that amplify a 1500 nucleotide region of the 18S rRNA gene from three major classes of algae: Bacillariophyceae, Eustigmatophyceae, and Chlorophyceae. "These amplicons can be sequenced for definitive identification of strains, or they can be digested with a restriction enzyme to generate allele-specific fragmentation patterns for rapid, inexpensive characterization of strains and cultures. This work provides molecular tools to detect and monitor algal population dynamics and clarifies the utility, strength, and limitations of these assays. These include tools to identify unknown strains, to routinely monitor dominant constituents in cultures, and to detect contaminants constituting as little as 0.000001% of cells in a culture. One of the technologies examined was shown to be 10,000X more sensitive for detecting weeds than flow cytometry" (Sayre et al., 2015). In addition, NAABB is also looking at developing molecular monitoring tools for tracking bacteria that are associated with the cultivation of different microalgal species as a means of determining the health of the culture and mitigating pond crashes.

Although some genera in the class Trebouxiophyceae can cause harmful algal blooms (HABs), such as the genus *Tetraspora*, *Chlorella* are not associated with harmful algal blooms (HABs). The genus is not listed as a harmful species, including in UNESCO's list of harmful micro algae (webpage: http://www.marinespecies.org/hab/ visited June 2017). The genus thrives in higher temperatures than other common species with moderate nutrient loaded environments so it is known to bloom later in the year (Elliot et al., 2006; Cordero et al., 2011). Although *Chlorella* has the potential of producing dense blooms, to date there is no available literature showing that *Chlorella* blooms have caused any adverse effects (Ryther, 1954). The only references that cite a *Chlorella* bloom event (ex. Pan et al., 2011; Li and Pan, 2013) are based on erroneous interpretation of a paper by Ryther (1954) who mentions *Chlorella* (but not in association with the observed decimation of the oyster industry on Long Island), which was actually attributed to eutrophication stimulated by duck farm effluents which led to blooms of *Nannochloris atomus* and *Stichoccocus sp.* So, to date, there has been no recorded HAB event associated with *Chlorella sp.*

However, one area of concern is the ability of some *Chlorella sp.* to produce chlorellin, an antibiotic-like substance that can inhibit its own growth and that of $Gram^+$ and $Gram^-$ bacteria. Older literature has demonstrated that *Chlorella* can produce substances that are inhibitory to the growth of other algae, such as *Nitzschia frustulum* (Rice, 1949). These experiments simply exposed competing algae to the exudates of *Chlorella sp.* and did not characterized the specific molecule(s) associated with the inhibitory effect. Therefore, it is possible that *Chlorella* may be able to outcompete other species if it is able to produce chlorellin or some other inhibitory molecule.

Potential effects of Chlorella sp. on terrestrial mammals

Indirect effects on terrestrial mammals can result from ecosystem-level disruptions through the establishment of novel strains of *Chlorella* in freshwater habitats. Disruptions of these freshwater ecosystems through the introduction of new algal strains could result in harmful algal blooms (HAB)

(Anderson et al., 2002). HAB events can disrupt highly complex stochastic mixing and flushing patterns and increase the eutrophication potential of waterways (Anderson, 2000; Hoagland et al., 2002). Disruptions of these waterways can negatively affect terrestrial wildlife that rely on freshwater ecosystems for food or habitat. However, as noted above, there is no literature indicating that *Chlorella* has ever been responsible for HABs.

Direct effects of *C. sorokiniana* on terrestrial mammals are not known, but effects from exposure to *Chlorella sp*., although rare, have been reported leading to infection of healthy tissues. Pathogenic infection of tissue by *Chlorella*, known as chlorellosis, has been reported in numerous species of mammals including gazelles, sheep (both adults and lambs), cattle, dromedaries, dogs and beaver (Cordy, 1973; Kaplan et al., 1983; Le Net et al., 1993; Philbey, 2001; Haenichen et al., 2002; Quigley, et al., 2009; Ramirez-Romero et al., 2010). Documented cases of chlorellosis are rare and are typically the opportunistic infections resulting from contamination of wounds or dissemination from the gastrointestinal tract following oral ingestion of stagnant water or sewage-contaminated water (Kaplan et al., 1983; Zakia et al., 1989; Philbey et al., 2001; Haenichen et al., 2002; Ramirez-Romero et al., 2010). Effects of chlorellosis in terrestrial mammals include the formation of lesions in the skin, liver, lungs and lymph systems accompanied by a characteristically green discoloration of the affected organs (Ramirez-Romero et al., 2010). Similar to infections in humans, ingestion of *Chlorella* has been shown to result in skin sensitivity, although organismal-level effects on terrestrial wildlife as a result of this effect are uncertain (Jitsukawa et al., 1984). While the majority of cases of chlorellosis have been reported in immunosuppressed individuals, several cases indicate that chlorellosis can occur in non-immunosuppressed mammals (Kaplan et al., 1983; Philbey et al., 2001). There is limited information available to characterize chlorellosis infections in terrestrial wildlife so there is uncertainty related to the mechanism of infection and which species of Chlorella are most likely to exhibit pathogenicity.

VII. POTENTIAL ECOLOGICAL HAZARDS OF THE SUBJECT MICROORGANISM

As mentioned previously, the *SNRK2* gene is expected and was shown by the submitters to help the proposed strain, PACE_Cs1412_SNRK2 have better growth and photosynthetic efficiency than wild-type recipient *C. sorokiniana* 1412. It has been reported in the literature that Arabidopsis *SNRK2*, when overexpressed, conferred increased sucrose synthesis, starch synthesis, and leaf growth (Zheng et al., 2010). The SNRK group of kinases have also been detected in almost all streptophyte algae (de Vries et al., 2018), and implicated with cold stress adaptation for the alga *C. reinhardtii* (Valledor et al., 2013). In preliminary experiments, the submitters report a 26-30% increase in growth (low to high light) (see Figure 3), along with a 21% increase in total carbohydrate accumulation in PACE_Cs1412_SNRK2 compared to wild-type *C. sorokiniana* 1412 (R-18-0001).

The growth characteristics of *Chlorella sorokiniana* has been extensively studied in literature due to its high performance in various factors (biomass, lipids, growth rate, and temperature tolerance) (Sayre et al., 2015). Many indoor/outdoor growth studies have been performed with this strain in attempts to optimize its productivity in various biotechnology fields. The genetic modifications presented for this submission enhances the growth and biomass accumulation of the submission microorganism, which can be viewed as increase in its competitive advantage in the environment as it will consume more nutrients at a faster rate than that of the wild type recipient.

Furthermore, the traits in PACE_Cs1412_SNRK2 are not new to the genus since increased growth and biomass accumulation have also been attained in wild type *C. sorokiniana* by tuning various growth parameters, which was reviewed by De Francisci et al. (2018). Table 3 shows that by adjusting basic

growth parameters, researchers can tune wild type *C. sorokiniana*'s growth rate, lipid content, FAME yield, and protein content. Based on these growth studies of *Chlorella* and the genetic modifications made to this strain, PACE_Cs1412_SNRK2 is expected to pose **low** ecological hazard.

VIII. POTENTIAL SURVIVAL OF THE SUBMISSION MICROORGANISMS IN THE ENVIRONMENT

The potential for survival of *Chlorella* was previously reported in the TERA risk assessment report for [redacted] As mentioned previously, *Chlorella* is one of the three most dominant green algae in many aquatic habitats and can be frequently isolated from marine, fresh water, soils and air samples, as they can tolerate a wide range of environmental conditions (Trainor, 1998). *Chlorella* is also a simple airborne microalga, present in terrestrial and aquatic habitats, whose minute cell size and resistance against environmental stress allows for long-distance dispersal (Hodac et al., 2016). *Chlorella* is an aerophilous algae (found in air), a type of algae shown to have better adaptation and growth responses compared to their solely soil and aquatic counterparts (Sharma et al., 2007).

In addition, Tiffany (1951), defined algae into nine different groups based on preferred habitat; including edapophytes (soil algae), aerophytes (aerial algae), endophytes (living within plant tissue) and endozoophytes (living inside animal hosts), all of which are habitats in which different *Chlorella* species have been known to thrive in. Lists of soil algae have been compiled across the country and the world, showing their diverse distribution, and frequently include *Chlorella* (Metting, 1981). Soil bound *Chlorella* species appear to tolerate high levels of radiation than other more complex terrestrial life forms (Metting, 1981). Trainor (1962) was even able to show that *Chlorella* is able to survive desiccation for one hour at 130˚C. Despite their high tolerance to a variety of stressors, Metting (1981) showed that various *Chlorella* strains are negatively affected by a variety of herbicides and insecticides, and thus could be used to minimize the dispersal of *C. sorokinana* cultured in outdoor ponds.

However, little research is available that directly shows that *C. sorokiniana* can survive in as well as many other species in the same genera, and more research is required on the wild type strain to determine the true potential for survival posed by new strain.

The survival characteristics are not expected to drastically change from the wild type recipient to the submission strain. Although the introduced genetic material does enable faster growth, it does not enable PACE_Cs1412_SNRK2 to survive in environments not tolerated by the wild type strain. In addition, the introduced *SNRK2* gene does not enable the submission strain with the ability to utilize any new or different substrates, nor does it impart any invasive properties.

IX. CONCLUSIONS

The recipient microorganism, *C. sorokiniana* DOE1412, was modified by the insertion of the *SNRK2* gene from *Picochlorum soloecismus* to produce the submission strain PACE_Cs1412_SNRK2. Since this genetic modification is only known to increase growth, photosynthetic efficiency, and biomass accumulation of the algae, it should not present any new hazards to the environment that is not already present with its wild type parent, as those desired effects can be attained by varying growth parameters of the wild type, which can occur naturally as environmental conditions change.